# Targeting Melanoma via the Innate Immune System

A Senior Honors Thesis

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By

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Abstract

Over-expression of the folate receptor (FR) has been shown on the vascular side of cancerous cells including those of the breast, ovaries, testes, and cervix. However, FR over-expression has not yet been explored fully in melanoma. We hypothesized that folate receptor is over-expressed on melanoma and that a novel folate-conjugated immunoglobulin G (F-IgG) would bind the FR to target melanoma cells for lysis by natural killer (NK) cells.

Folate receptor expression was confirmed in the Mel-39 (human melanoma) cell line by flow cytometry and immunoblot analysis, using KB (human oral epithelial) and F01 (human melanoma) as a positive and negative control, respectively. FR-positive and negative cell lines were coated with F-IgG or control immunoglobulin G (C-IgG) in the presence or absence of cytokines in order to determine NK cell ability to lyse FR-positive cell lines. NK cell activation was significantly upregulated and lysis of Mel 39 tumor cells enhanced following treatment with F-IgG, as compared to C-IgG at all effector:target (E:T) ratios ($p<0.01$). This trend was further enhanced by the addition of interleukin-12 (IL-12). NK cell production of cytokines such as interferon-gamma (IFN-$\gamma$), macrophage inflammatory protein 1 alpha (MIP-1$\alpha$), and regulated on activation normal T-cell expressed and secreted (RANTES) were also significantly increased in response to IL-12 stimulation and F-IgG-coated Mel 39 target cells, as compared to controls ($p<0.01$). In contrast, F-IgG did not bind to F01 (FR-negative cell line) and had no significant effect on NK cell lysis or cytokine production. This research indicates the potential use of F-IgG for its ability to induce an immune response from NK cells against FR-positive melanoma tumor cells which can be further enhanced by the addition of cytokines.

Specific Aims

**Aim 1:** To test the hypothesis that FR is expressed in melanoma cells lines. ***Rationale 1:*** It has been shown that FRs are over-expressed in various cancers, including cancers of the cervix, ovaries, breast, uterus, pituitary gland, lung, brain, and testes (3, 4). However, there is limited data indicating FR expression in melanoma tissue. Therefore, we propose to test if the FR is expressed in melanoma tumor cell lines. In this specific aim, FR expression will be assessed utilizing reverse transcriptase polymerase chain reaction (RT-PCR) and immunoblot analysis, *in vitro*.

**Aim 2:** To test the hypothesis that F-IgG binds to FR-positive melanoma cancer cells.

***Rationale 2:*** Our lab has demonstrated the ability of F-IgG to bind the FR in multiple cancer cell lines, *in vitro*. In this specific aim, we propose to examine the ability of F-IgG binds to melanoma tumor cell lines by utilizing flow cytometric analysis, *in vitro*.

**Aim 3:** To test the hypothesis that F-IgG-coated melanoma tumor cells result in NK cell activation.

***Rationale 3:*** Our lab has demonstrated that F-IgG-coated human oral epithelial and murine leukemia tumor cells promote increased NK cell killing and cytokine secretion, as compared to C-IgG-coated tumor cells. Therefore, it will be determined if F-IgG-treated melanoma tumor cells promote increased NK cell killing and cytokine secretion. In this specific aim, the ability of F-IgG to enhance NK cell killing will be assessed utilizing antibody-dependent cell-mediated cytotoxicity (ADCC) and activation will be measured by enzyme-linked immunosorbent assay (ELISA) as well as flow cytometric analysis, *in vitro*.

Introduction

**Melanoma:** Cutaneous melanoma (CM) arises from neural crest-derived epidermal melanocytes found next to the basal layer of the epidermis whose main role is the production of the pigment melanin (5). The epidermis is only a thin layer of skin cells; however, it has a large role in protecting underlying cells and organs from exterior factors such as damaging ultraviolet light due to the presence of melanin (**Figure 1**). The genesis and development of melanoma has yet to be fully understood on a molecular basis; however, there have been many genetic mutations previously described that may result in its occurrence (6). Consequentially, a wealth of information has yet to be gathered regarding CM and the way in which it grows and metastasizes.

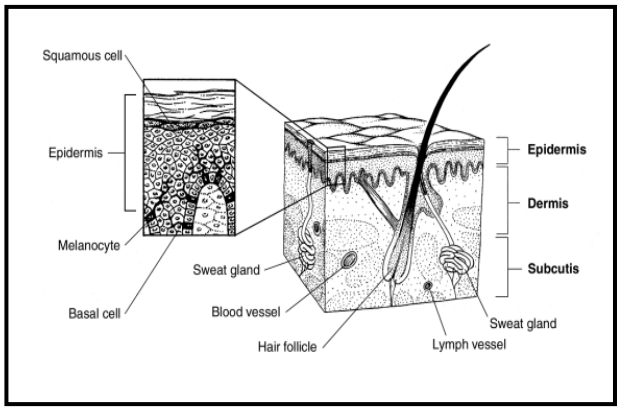


**Figure 1**. Dermis Layers (1).

Additionally, mortality rates of people with CM have continued to climb despite increased awareness, research, and public education. The World Health Organization has estimated there will be 120,000 new cases of CM per year internationally; this estimate has been on the rise (7). Presently, over 79% of skin cancer related deaths are attributed to CM and culminate in, on average, one American dying from CM almost every hour (8). While a staggering number of people are expected to be diagnosed with CM yearly, few patients, such as those with metastasized melanoma or melanoma of the lymph node, will respond to surgical procedures or systematic therapy long term. Melanoma cells appear to be fundamentally

resistant to previously proposed drugs, such as Temodar and Yervoy, as well as radiation therapy.

**Folate:** Folate is a water soluble B vitamin used for cellular growth and associated processes. Specifically, folate functions as a co-factor in various cellular metabolic processes of the cell such as nucleic acid and protein biosynthesis. Leafy green vegetables, legumes, egg yolks, and fresh fruit are natural sources of folate. However, the common dietary source of folate is achieved through supplementation and food fortification, where the vitamin is present in its oxidized form known as folic acid (5, 9). Additionally, folic acid is hypothesized to increase cancer cell motility, invasion, metastases and overall tumor progression (10-12).

**Folate receptor:** Cellular folate uptake is mediated by at least three distinct transporters which include the reduced folate carrier (RFC), a proton-coupled high affinity folate transporter, and the folate receptor (FR) (13). The RFC is a bidirectional low affinity transmembrane anion carrier that is the predominant folate transporter in most cells and is ubiquitously expressed on normal tissues. The RFC will not transport folic acid (oxidized form) or folate conjugates (14). The second transporter of folate, the proton-coupled high affinity transporter, is a major folate transporter in low pH environments and appears to be the principal transporter in intestinal absorption of folate. The FR is a membrane bound high affinity unidirectional folate transporter protein that characteristically binds folic acid, chemical conjugates of folic acid, as well as folate-linked immunological agents (8). The FR has three well-established isoforms, α, β, (both linked to glycosylphosphatidylinositol; GPI) and γ (15).

Specifically, in normal epithelial cells, the FR is located mainly on the luminal side of the cell and is consequentially largely inaccessible to circulating folate. In contrast, the FR of malignant cells, such as lung, uterus, pituitary gland, cervix, ovaries, ependymal brain, and blasts of myeloid leukemias, is over-expressed on the extracellular side of the cell and therefore available to bind circulating folate (3, 16-22). This altered expression is not well understood, however, it is hypothesized that this occurs in order to supply higher levels of folate to malignant cells for enhanced cellular growth. Estimates suggest that roughly 1/3 of all cancers may involve the up-regulation of the FR, with metastatic and later-stage cancers associated with increased FR over-expression compared to the FR expression of non-malignant cells (23).

**Natural killer (NK) cells:** NK cells are lymphocytes, a major component to the innate immune system, that defend the body against foreign substances. They have the capacity to lyse malignant cells with altered expression of major histocompatibility antigens by virtue of their expression of killer cell immunoglobulin receptors (KIR). They also express numerous cellular adhesion molecules, multiple receptors for stimulatory cytokines (i.e. interferon-gamma [IFN-γ], tumor necrosis factor-alpha [TNF-α], and macrophage inflammatory protein 1 alpha [MIP-1α]), possess cytolytic granules that contain perforin and granzymes, and produce cytokines with anti-tumor effects. Additionally, NK cells express FcγRIIIa, an activating receptor that recognizes the constant (Fc) region of Immunoglobulin G (IgG). The FcγRIIIa receptor is critical in mediating antibody-dependent cell-mediated cytotoxicity (ADCC) against antibody (Ab)-coated targets (3, 24).

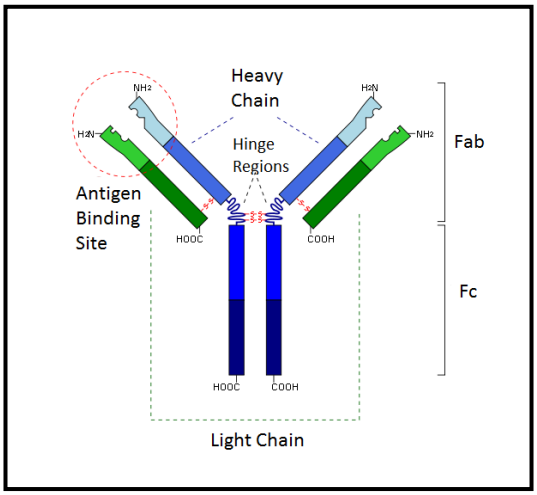


**Figure 2.** Simplified diagram of human antibody structure.(2)

**Constant region (Fc):** IgG are 150kDa antibody molecules made of two heavy chains, two light chains and two different binding sites, as seen in Figure 2. Disulfide bonds hold together the heavy and light chains. The end of each Fab region contains an antigen binding site whilst the Fc region of the molecule, also known as the constant region, is recognized by immune cells. When treating FR-positive tumor cells with the folate-conjugated immunoglobulin G (F-IgG), the cell effectively becomes coated with IgG, allowing cells of the immune system with Fc receptors (FcR) to target the Fc portion of the IgG and destroy these cells. Previous research has demonstrated that NK cells contain FcRs that bind antibody-coated cells and target them for killing via ADCC (25-28). It is also well established that NK cells are crucial mediators of ADCC via perforin and granzyme lysis of tumor cells. Furthermore, the interaction between NK cells and antibody-coated tumor cells has been demonstrated *in vitro* and *in vivo* and results in overall decreased tumor growth (29-36).

**Rationale for the use of a F-IgG conjugate:** The FR has the potential to be exploited as a valid therapeutic target. This is due to its over-expression on tumor cells, as well as the fact that receptor endocytosis does not lead to lysosome-mediated destruction of folate-based therapeutics. This allows the folate therapeutic to enter the cell and avoid destruction because of the requirement for folate by the cell (37). The FR itself is efficiently recycled and returned to the cell surface, allowing for the re-uptake of additional folate and foltate conjugates. These

parameters are thought to create a favorable toxicity profile for folate-conjugated anti-tumor compounds (4, 13, 37-40).

The utilization of the FR has also been exploited in several other studies, which demonstrate the importance of this transport mechanism. Other studies have targeted the FR utilizing multiple methods, such as monoclonal antibodies, folate-liposome loaded delivery and folate-FITC targeting (4, 41). This data, along with our own studies, support the development of FR-directed therapeutic immunoconjugates, where the FR serves as a ligand for cancer-specific targeting.

Materials and Methods

**Human F-IgG synthesis.** The technique for conjugating folate to macromolecules has been previously described (14, 42). Folate was reacted with N, N'-dicyclohexylcarbodiimide (DCC) and N-hydroxysuccinimide (NHS) in DMSO at stoichiometric molar ratios of 1:1:1 for 3 hrs at room temperature. The resultant product was filtered to remove precipitate. Then activated folate (f-NHS) was reacted with IgG (Equitech-Bio Inc., Kerrville, TX) in pH 8.0 PBS buffer solution at a molar ratio of 12:1 for 2 hrs at room temperature. After incubation, to remove unreacted f-NHS, the sample was passed through a PD-10 column equilibrated in PBS (pH 8.0). For the preparation of FITC labeled F-IgG (F-IgG-FITC) or IgG (IgG-FITC), activated f-NHS was reacted with IgG and FITC in pH 8.0 PBS buffer solution at a molar ratio of 12:1:5 for 2 hrs at room temperature. The folate content of the construct was determined by UV spectrometry at 371 nm and the antibody concentration was determined by the BCA protein assay method. Conjugation reactions performed at IgG-to-folate-NHS ratios of 12:1 and 100:1 yielded preparations containing an average molar ratio of 2.6 folate molecules per IgG molecule and 9.0 folates per IgG, respectively. Since excessive folate conjugation may negatively affect the effector function of the IgG, a lower conjugation level of 3 folates per IgG was chosen for further study. Unmodified human IgG molecules were used as controls (C-IgG). Conjugates were stored at 4°C in the dark prior to use.

**Cell lines.** The FR-positive human cell lines Mel-39 (human melanoma, ATCC) and KB (human oral epithelium, a gift from Dr. Philip S. Low, Purdue University, West Lafayette, Indiana), as well as the FR-negative cell line F01 (human melanoma) were propagated in folate-free RPMI 1640, supplemented with 10% heat-inactivated fetal bovine serum (FBS), 1% antibiotic-antimycotic, and 0.1% plasmocin (Gibco).

**Isolation of human NK cells.** NK cells were isolated directly from fresh peripheral blood leukopacks (American Red Cross, Columbus, OH) by 30-minute incubation with RossetteSep cocktail (Stem Cell Technologies, Vancouver, BC) prior to Ficoll Hypaque (Sigma) density gradient centrifugation. Human NK cells were cultured in folate-free RPMI 1640 (purchased from The Cleveland Clinic Media Preparation Service, Cleveland, OH) supplemented with 10% heat-inactivated pooled human AB serum (HAB; C-Six Diagnostics, Germantown, WI) and 1% antibiotic-antimycotic.

**Reverse Transcription Polymerase Chain reaction (RT-PCR) for FR-α and FR-β expression.** Total RNA from Mel 39, KB, and F01 cells was extracted in TRIZOL® Reagent using the RNeasy Mini Kit (Qiagen, Valencia, CA) following the manufacturer's protocol. RNA was converted to complementary DNA by reverse transcription and used as a template for RT-PCR using FR-α (forward primer, TGGGTGGCTGTAGTAGGGGAG; reverse primer, CAGGGGCACGTTCAGTACC) and FR-β primers (forward primer, ACCAATGCAGTCC-CTGGAAGAAGA; reverse primer, AGCTGGGCACTTGTTAACTCCTGA) (33). The PCR conditions consisted of 95°C for 30s followed by 30 cycles of 95°C for 30s, 58°C for 30s, and 72°C for 40s.

**Flow Cytometric Analysis.** The FR-positive cell lines, Mel 39 and KB, or the FR-negative cell line, F01, were grown in folate-free RPMI 1640, supplemented with 10% heat-inactivated fetal bovine serum (FBS), 1% antibiotic-antimycotic, and 0.1% plasmocin. 1 x $10^6$ cells were resuspended in 500 µL of media in flow tubes and stained with 20 µg of C-IgG-FITC or F-IgG-FITC. Cells were incubated for 1-hr at 4°C, washed with 1mL PBS with 1% TBST (flow buffer) at 1700 rpm for 10 min. Cells were then fixed with 300 µl of 1% formalin in PBS after

aspirating the supernatant and examined via flow cytometric analysis, as previously described (32).

**Cytotoxicity assays.** Purified human NK cells were plated in 96-well V-bottom plates in 10% HAB containing folate-free RPMI 1640 medium supplemented with interleukin-12 (IL-12) (10 ng/mL), and incubated overnight at 37°C. For ADCC assays, F-IgG or C-IgG-treated (100 ug/mL) $^{51}$Cr-labeled target cells were added to NK cells at different effector:target (E:T) ratios. Following a 4-hr incubation at 37°C, supernatants were harvested for quantification of chromium release. Percentage of lysis was determined as previously described (35).

***In vitro* co-culture assay.** The FR-positive cell lines, Mel 39 and KB, or the FR-negative cell line, F01, were cultured in the wells of a 96-well flat-bottom culture plate overnight at 37°C, as previously described (29). Briefly, the culture supernatant was aspirated the following day and wells were treated with 100 µg/mL F-IgG or C-IgG for 1 hr at 37°C. After washing off unbound F-IgG or C-IgG, purified NK cells were then added at 2 x $10^5$ cells per well in 200 µL of folate free RPMI containing 10% HAB medium and 10 ng/mL IL-12. Control conditions consisted of NK cells plus tumor cells treated with medium alone, F-IgG or C-IgG alone, or cytokine alone. Culture supernatants were harvested after 48 hours and analyzed for IFN-γ, MIP-1α, and regulated upon activation, normal T-cell expressed and secreted (RANTES) content by enzyme-linked immunosorbent assay (ELISA). All results shown are the mean of triplicate wells ± SD. Purified NK cells were co-cultured with Mel 39 tumor cells and collected after 48 hours, then examined for activation. Harvested NK cells were incubated with mouse-anti-human CD56-APC and CD69-PE antibodies (BD Pharmingen) as directed by the manufacturer. Samples were then washed with flow buffer and fixed in 1% formalin in PBS and assessed via flow cytometric analysis as previously described (32).

**Statistics.** These experiments tested whether there were significant effects with F-IgG alone and synergistic effects with IL-12 on NK cell mediated ADCC and cytokine production. A student's t-test and an analysis of variance (ANOVA) were utilized for two-way and multiple comparisons, respectively.

Results

*The FR is expressed on melanoma tumor cell lines.*

To evaluate whether F-IgG has a FR to bind to, we analyzed the expression of the FR on various melanoma cell lines via RT-PCR and western blot analysis. RT-PCR was used for its ability to provide confirmation of the presence of FR RNA sequences. Western blot analysis allowed for the characterization of FR protein content of the various malignant cell lines. FR was over-expressed on Mel 39 (human melanoma) and KB (human oral epithelial), but not on F01 (human melanoma) malignant cell lines via both RT-PCR and Western blot analyses **(Figure 3A-B)**. The KB and F01 tumor cells lines served as positive and negative controls, respectively. It is important to note that, as seen by the F01 cell line, that not all melanoma express a FR + profile.

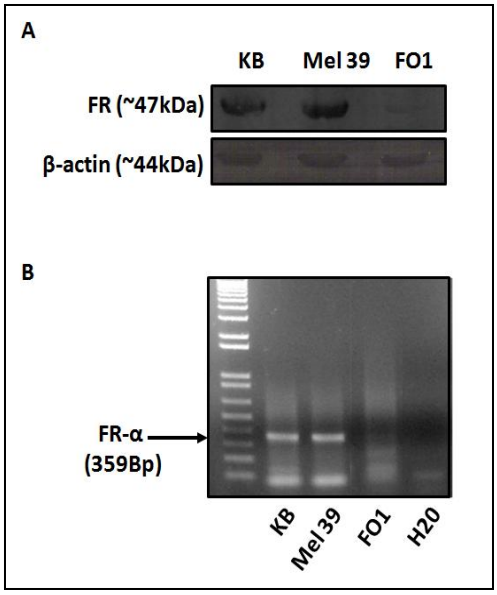


**Figure 3**. FR expression in melanoma cell lines. (A)Western Blot and (B) PCR analyses of FR expression in melanoma cell lines.

*F-IgG binds to FR-positive melanoma tumor cells.*

It was hypothesized that F-IgG binds to FR-positive melanoma tumor cell lines with great affinity. Flow cytometry was utilized to assess binding of F-IgG. Here, a F-IgG-FITC conjugate was utilized. This method was used for its ability to demonstrate the presence of the FR on the surface of

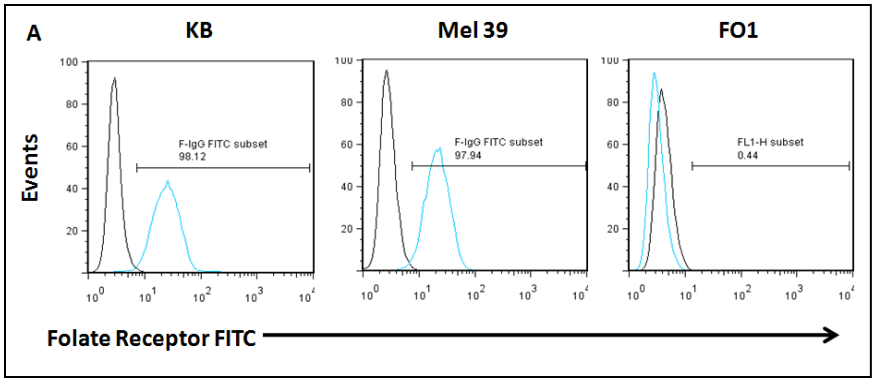


**Figure 4**. F-IgG binds FR (blue) in melanoma cell lines. (A) Flow cytometric analyses of F-IgG binding to FR in melanoma cell lines.

the various malignant cell types and to determine if the F-IgG conjugate binds to the surface of these cells. The Mel 39 and KB cell lines were >97% FR-positive, as indicated by F-IgG-FITC flow cytometry. This was not observed with the FR-negative cell line F01, which was <1% FR-positive (**Figure 4**).

*NK cells are activated by F-IgG coated FR-positive melanoma tumor cells.*

It was hypothesized that NK cells would be activated in response to F-IgG coated FR-positive melanoma cell lines. To determine the ability of F-IgG enhancement of NK cell activation, ADCC and ELISA assays were utilized, *in vitro*. In support of this hypothesis, it was found that F-IgG coated FR-positive cell lines resulted in NK-cell mediated ADCC. A four-hour $^{51}$Cr-release assay was utilized, where malignant cells were labeled with the radioactive isotope, coated with the F-IgG, then exposed to NK cells at different effector:target (E:T) ratios. During this experiment, NK cells served as effector cells and Mel 39, KB, or F01 served as the targets. NK cell lysis of F-IgG-coated Mel 39 target cells was significantly enhanced, as compared to C-IgG ($p<0.01$). This was further increased following treatment of NK cells with IL-12 (10ng/mL). IL-12 activated NK cells lysed 50±5% F-IgG-coated Mel 39 tumor cells as compared to <20% lysis for the control groups

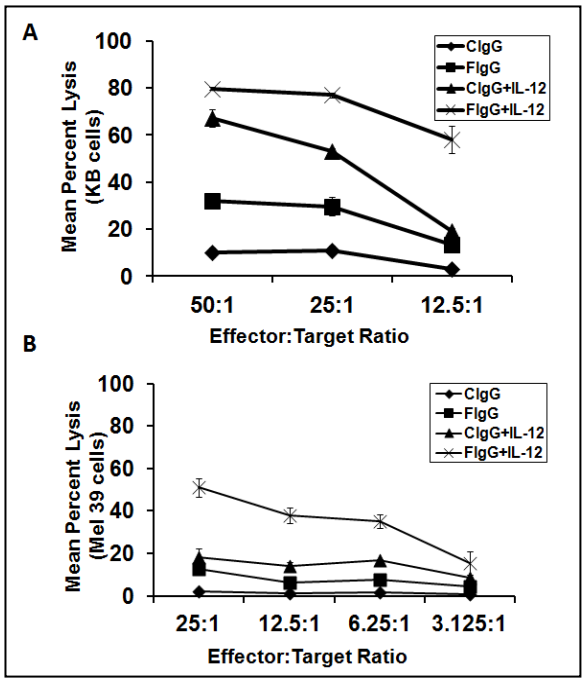


**Figure 5**. F-IgG coated FR-positive melanoma cells activate NK cells. (A) Four-hour 51Cr-release release ADCC results for NK cell lysis of F-IgG+IL-12, C-IgG+IL-12, F-IgG, or C-IgG coated KB tumor cells.

(**Figure 5A-B**). Notably, the FR-negative cell line, F01, demonstrated < 20% lysis for all treatment groups (data not shown).

NK cell activation was also assessed by cytokines secreted in this setting. Here, NK cells were exposed to F-IgG coated melanoma tumor cells. NK cell production of IFN-γ, RANTES, and MIP1-α was significantly enhanced in response to F-IgG-coated Mel 39 target cells, as compared to C-IgG ($p<0.01$). This effect was further enhanced by IL-12. IL-12 activated NK cells exposed to F-IgG-coated FR-positive melanoma tumor cells produced 7100 pg/mL IFN-gamma at 72 hours as opposed to < 1200 pg/mL for the control conditions (C-IgG, C-IgG + IL-12, or F-IgG). Similarly, the NK cell production of RANTES and MIP1-alpha was significantly and synergistically enhanced by FIgG alone and F-IgG + IL-12, respectively ($p<0.01$) (**Figure 6 A-C**).

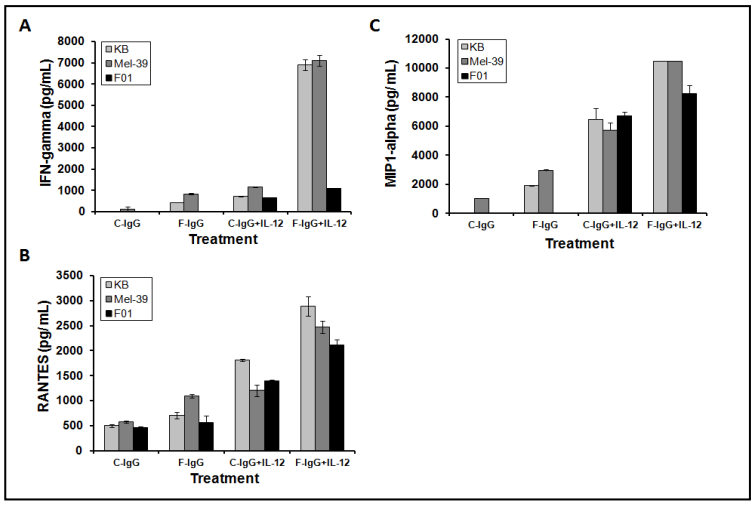


**Figure 6**. NK cell IFN-γ (A), RANTES (B), and MIP1-α (C) cytokine secretion in response to C IgG and F-IgG coated tumor cells.

NK cell activation was further demonstrated by changes in NK cell surface markers. C-IgG or F-IgG coated Mel-39, F01, and KB tumor cells were co-cultured with and without IL-12 stimulated NK cells in a 96-well plate. After 48 hours, cells were harvested and stained for

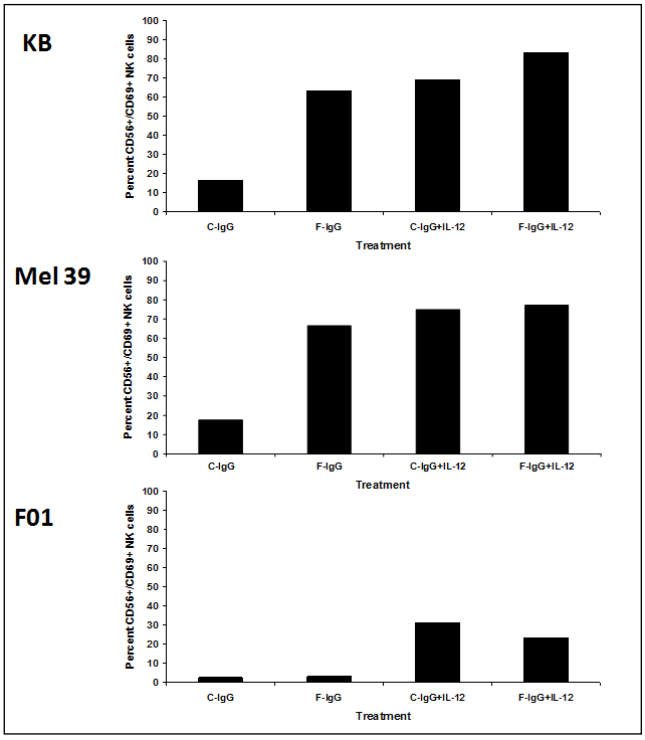


**Figure 7**. NK cells are activated in response to F-IgG and F-IgG+IL-12.

CD56+ (identifying NK cells) and CD69+ surface markers to assess NK cell activation. NK cells co-cultured with KB and Mel 39 had increased NK cell activation, with the greatest activation following dual stimulation. This trend was not observed with the FR-negative, F01, melanoma cell line **(Figure 7)**.

Discussion

Previous studies involving the FR have indicated the presence of FR in many cancers, including those of the ovaries, breast, and cervix (9). These studies also noted the potential exploitation of FR over-expression in possible cancer therapeutics. The presence of FR in human melanoma, however, has not been fully characterized or defined. This study determined the presence of FR in melanoma tumor cell lines in order to assess the potential therapeutic application of a F-IgG conjugate in this setting.

Here, it was found that FR over-expression was significantly higher in the Mel 39 melanoma cell line, compared to other non-malignant cell lines. This indicates the opportunity to exploit the FR as a selective target for melanoma. Other melanoma cell lines will be explored by our laboratory, as this would be necessary to test the statistical significance of the observation that the FR is over-expressed in melanoma cell lines.

These results indicate that F-IgG is able to bind to FR-positive Mel 39 melanoma tumor cells. This data demonstrates the potential use of F-IgG as a selective therapeutic in melanoma as well as other FR-positive cancers cell lines. Since FR over-expression has been correlated with malignancy, this potential therapeutic allows for the selective targeting of FR-positive melanoma cells as compared to other nonmalignant cells present.

Furthermore, we demonstrate that F-IgG coated FR-positive melanoma cell lines are able to stimulate an immune response through NK cell-mediated ADCC and enhanced activation. Specifically, increased tumor cell killing might occur in an *in vivo* setting where there may be a more significant anti-tumor immune response at the tumor site. NK cell cytokine secretion will, in turn, support the infiltration of other immune cells via the enhanced NK cell cytokine production observed. IFN-γ, MIP1-α, and RANTES are well known inflammatory cytokines that

are involved in mediating the migration of various immune cells which are integral to the anti-tumor response in an *in vivo* setting (31).

From the results of our study we conclude that FR over-expression is found in Mel 39 melanoma tumor cells and that F-IgG is able to bind to these cells and stimulate an immune response which is further enhanced by IL-12 addition.

## Significance

Research into FR expression on melanoma cells is critical in the development of selective targeting of malignant cells with minimal effects on nonmalignant cells. With the data obtained in this study, the potential use of F-IgG as an anti-tumor compound has been supported due to the ability of F-IgG to stimulate an anti-tumor response in FR-positive melanoma cell lines. The observations of this study provide a greater understanding of this possible therapy and its effects on NK cells which may next be translated into an animal model and eventually the clinic.

## Acknowledgments

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